Chemical Analysis for Antibiotics Used in Agriculture

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Hiroyuki NAKAZAWA

Department of Analytical Chemistry,
Faculty of Pharmaceutical Science, Hoshi University

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Introduction

The Japanese life expectancy has increased every year, with both male and female Japanese enjoying the longest life spans in the world. In 1991, the average life expectancy for Japanese men was 76.11 years and for Japanese women was 82.11 years. The primary reason for this increase is the diversification of the Japanese diet. Also, there has been a significant change since World War II when Western foods were introduced and quickly became part of the Japanese diet. As a result, the consumption of rice has declined and dishes based on meat and dairy products have become popular. Young people, in particular, prefer western style meals, in which meat is a major ingredient as compared to the traditional Japanese style foods. As a result, the livestock industry in Japan has prospered.

Outlook of Livestock and Fishery Industries

Livestock production accounted for 27% of Japan's gross agricultural output in 1991 and outperformed the 25% figure set by rice production. In 1992 there were approximately 4.9 million cattle (including both dairy and beef), 10.9 million pigs, 336.2 million fowl (including broilers and layers), 29 thousand sheep, 35 thousand lambs, and 26 thousand horses in Japan as shown Fig. 1[1]. The breeding scale in the livestock industry has been enlarged and has become more intense on reducing operating expenses from year to year. While the number of livestock breeders has generally been increasing, the number of farming households has been decreasing annually. The following statistics for the type and number of farms in 1992[1] are followed by the rate of decline relative to 1991 and are indicated in parentheses: dairy farms, 55,000 (8%); beef cattle farms, 210,000 (5%); pig farms, 30,000 (17%); egg farms, 9,200 (9%); and chicken farms, 4,700 (7%). This reduction in the total number of each type of livestock farms is a result of the increasing age of farmers and their lack of successors for these farms and is especially prevalent among small- to medium-sized farms. In contrast, the number of large-scale livestock producers has increased, and their output per facility has been growing. For example, Fig. 2 illustrates the trend towards large

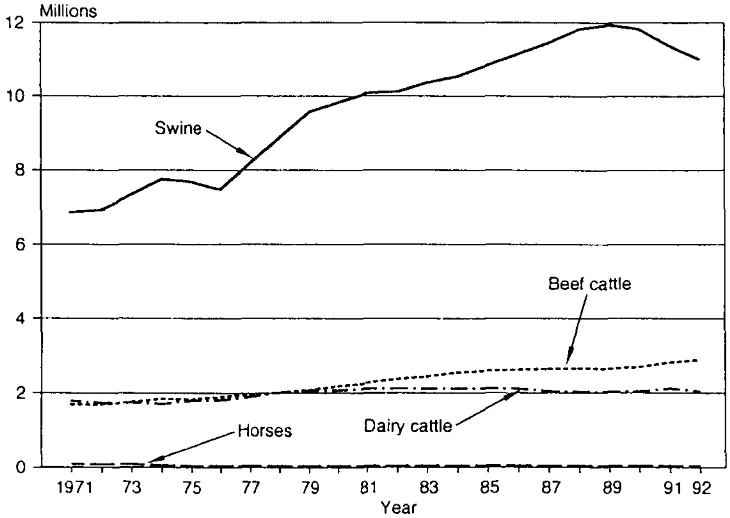


Fig. 1. Number of livestock in Japan.

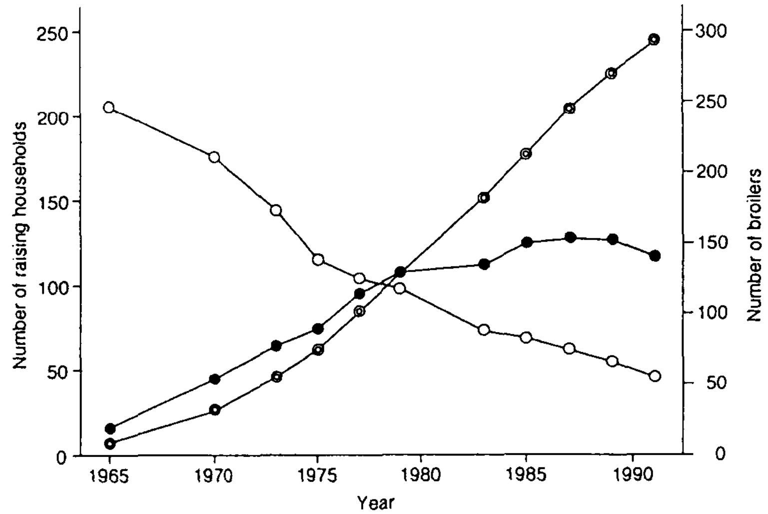


Fig. 2. Comparison of annual population of broilers and number of raising households.
○: Number of raising households (×100)
●: Total number of broilers (×1,000,000)
◎: Number of broilers per one farm (×100)

scale broiler breeding. On a typical chicken farm, birds are reared on a conveyor system, continuous feeding, and with no sunshine or exercise.

Although the average Japanese intake of meat has increased, the consumption of fish remains high when compared to the consumption of fish in other countries, as shown in Fig. 3[1]. Marine products provided a daily supply of 18.4 g of animal protein per capita in 1991, accounting for 40% of the total protein supply (45.6 g)[2]. Japan, which had been the world leader in fisheries production for many years, slipped to second place in 1989. In 1990 the Japanese accounted for

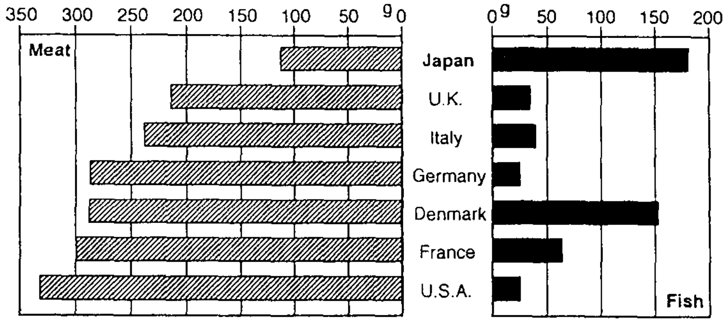


Fig. 3. The amount of consumption of meat and fish of principal countries .
Source: Ministry of Agriculture, Forestry and Fisheries; "Food Comsumption Statistics," OECD.
Note: Japan 1991, others 1988.

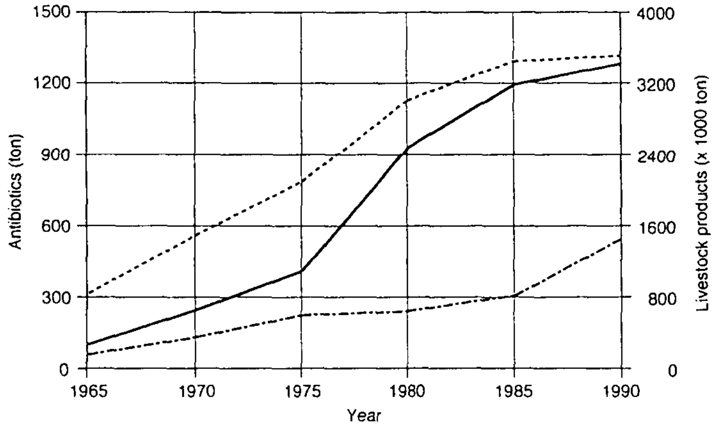


Fig. 4. Production amount of antibiotics as veterinary drugs (——) and live stock products (domestic: ·······, imported: -·-·-).

10.9% of the world's production of 101.58 million tons of fish. The total fish catch in 1991 was 9.98 million tons, 1.07 million tons less than the previous year. In per capita terms, the supply of fish and shellfish in 1991 decreased by 4.5 kg to 66.7 kg, compared to 1990. Factors contributing to this decline from 1989 to 1990 included a decrease in domestic catch, the scale of supply–demand for fish and shellfish, intensified restrictions on international fishing operations, and increased fishery imports, all of which contributed to a decrease for three consectuive years from 1989. In addition, the source of fisheries production in Japan has also changed, with the production of the marine culture, or aquaculture, industry increasing over the last 20 years. Concern over these issues in Japan has recently focused public attention on the aquaculture industry.

The livestock and fisheries industries which feature intensive practices, maintain a large population in a small area where the incidence of disease can result in a heavy economic loss. To decrease the cost of production and to improve the quality of livestock and fishery products while raising productivity, various compounds have proven effective against disease, including anti-

Table 1. Outlook of Feed Attitives Used in Japan

Use	Classification	Designated feed additives
Prevention of quality deterioration	Antioxidants (3)[a] Antifungal agents (3) Emulsifiers (4)	BHA, BHT, Ethoxyquin Propionic acid and its Na and Ca salt Propyleneglycol
Dietary supplements	Amino acids (7) Vitamins (28) Minerals (32)	DL-Alanine, L-tryptophan L-Ascorbic acid, thiamine hydrochloride
Promotion of effective utilization of nutritional ingredients in feed	Synthetic antibacterials (7) Antibiotics (21)	Amprolium, ethopabate, sulfaquinoxaline Avoparcin, tylosin, tetracycline

[a] The numbers in parenthesis indicate the feed additive numbers.

biotics used as feed additives and veterinary drugs. Fig. 4 shows that the production of veterinary drugs, such as antibiotics, has increased in proportion to the increase in livestock production. Because antibiotics are officially inspected before marketing, the quantities produced for livestock use can be estimated. In 1991, approximately 58.9 tons[3] of antibiotic assays for animal use and veterinary pharmaceuticals sold, including antibiotics, had a market value of 75 billion Yen[4].

Outline of Feed Additives and Veterinary Drugs

Table 1 lists the feed additives used in Japan, which are divided into three categories according to their usage. The first group, comprised of antioxidants and antifungal agents, is used to prevent the quality of feed from deteriorating. The second group, which includes 7 amino acids, 28 vitamins, and 32 minerals, is used as dietary supplements. To improve the efficiency of feed utilization, a third group consisting of 7 synthetic antibacterials (such as amprolium and sulfa drugs) 7 and 21 antibiotics (avoparcin, tetracycline, etc.) has been used as feed additives. These substances are specific in the Law Concerning Safety Assurance and Quality Improvement of Feed in Animal Husbandry and Aquaculture (Government Ordinance No. 68 of 1976).

The principal legal regulation dealing with feed additives is the Pharmaceutical Affairs Law (Law No. 145) established in 1960. Veterinary drugs are intended exclusively for use in animals. Table 2 summarizes the antibiotics currently regulated as feed additives and veterinary drugs by the Pharmaceutical Affairs Law and the Law Concerning Safety Assurance and Quality Improvement of Feed. Synthetic antibacterials used as veterinary drugs and feed additives have been the subject of a recent review[5]. Table 3 gives a brief comparison of feed additives and veterinary drugs. It indicates that feed additives are used at trace levels for a relatively long period of time for promoting growth, while veterinary drugs are prescribed at relatively large dosages over a short period of time for preventing and treating infectious diseases.

Legal Restrictions on Drug Residues in Livestock Products

Table 4 indicates the approved dosage of the antibiotics used in the treatment of fish diseases. In the case of spiramycin, for example, the drug is given in feed at 40 mg/kg of fish weight per day to treat streptococcists in yellowtail. Table 4 also shows that a period of 30 days is required by law between the time of final treatment and the time of harvesting the fish for human consumption.

Table 2. Antibiotics Regulated as Veterinary Drugs, Feed Additives, or Both

Class	Antibiotics	Veterinary drugs	Feed additives
β-Lactams	Amoxicillin	*	
	Ampicillin	*	
	Cloxacillin	*	
	Dicloxacillin	*	
	Mecillinam	*	
	Nafcillin	*	
	Penicillin G	*	
	Cephalonium	*	
	Cephazolin	*	
Aminoglycosides	Apramycin	*	
	Destomycin A	*	*
	Dihydrostreptomycin	*	
	Fradiomycin	*	
	Gentamicin	*	
	Hygromycin B	*	*
	Kanamycin	*	
	Spectinomycin	*	
	Streptomycin	*	
Tetracyclines	Chlortetracycline	*	*
	Deoxytetracycline	*	
	Oxytetracycline	*	*
	Tetracycline	*	
Macrolides	Erythromycin	*	
	Josamycin	*	
	Kitasamycin	*	*
	Mirosamycin	*	
	Oleandmycin	*	
	Sedecamycin	*	
	Spiramycin	*	
	Terdecamycin	*	
	Tylosin	*	
Polypeptides	Avoparcin		*
	Bacitracin	*	*
	Colistin	*	*
	Enramycin	*	*
	Noshiheptide		*
	Thiopeptin	*	*
	Virginiamycin	*	*
Polysaccharides	Flavophospholipol	*	*
Polyethers	Lasalosid		*
	Monensin	*	*
	Salinomycin	*	*
	Avilamycin		*
Others	Bicozamycin	*	*
	Chloramphenicol	*	
	Fosfomycin	*	
	Lincomycin	*	
	Novobiocin	*	
	Polynactin		*
	Tiamulin	*	

[a] Veterinary drugs: regulated by the Pharmaceutical Affairs Law.
[b] Feed additives: regulated as feed additives in the Law Concerning Safety Assurance and Quality Improvement of Feed.

Table 3. Comparison of Feed Additives and Veterinary Drugs

	Feed additives	Veterinary drugs
Application	Growth promotion	Prevention and treatment of infectious diseases short
Period	Long (2–3 months)	
Dose	Trace	Relatively large amount
Regulation	SAQIF	Pharmaceutical Affairs Law
Synthetic antibacterials	Sulfaquinoxaline, clopidol, nicarbazin, olaquindox (7)	Sulfadimidine, carbadox, oxolinic acid etc. (ca. 25)
Antibiotics	Chlortetracycline, lasalosid etc. (21)	Ampicillin, kanamycin etc. (ca. 50)

Table 4. Application and Dose of Antibiotics Used in the Treatment of Fish Diseases

Active drugs	Fish	Disease	Application and dose	Day[a]
Spiramycin	Yellowtail	Streptococcisis	40 mg/kg of fish weight per day in feed	30
Oxytetracycline hydrochloride	Red sea bream	Vibriosis	50 mg/kg of fish weight per day in feed	3
Oxytetracycline hydrochloride	Kuruma shrimp	Vibriosis	50 mg/kg of shrimp weight per day in feed	25

[a] Period required by law for stopping treatments before harvesting for human consumption.

In recent years, public concern over the presence of drug residues in meat products has grown rapidly in Japan. To prevent the occurrence of veterinary drug residues, the law prescribes that animals are not to be slaughtered shortly after the drugs are administered or while the concentration of the drugs remains at therapeutically effective levels. However, instances of illegal or extra-label uses of these drugs are occasionally found.

Residual Analysis of Feed Additives and Veterinary Drugs in Livestock Products

Another essential law is the Japanese Food Sanitation Law (Law No. 233) established in 1947, which describes standards and criteria of foods. According to this law, no food should contain antibiotics and meat, poultry, eggs, fish, shellfish, milk, and dairy products should not contain any synthetic antibacterial substances. Based on these legal regulations, it is necessary to analyze foods for residues of synthetic antibacterials and antibiotics used as feed additives or veterinary drugs. For this purpose, the screening techniques used should be relatively simple, rapid, and inexpensive and permit a large number of samples to be analyzed with high reliability. It is also necessary to use a different analytical technique to confirm the presence of a detected residue. The analytical methods approved by the Japanese Government are published as two volumes. Volume 1 contains the microbial inhibition tests organized by the Veterinary Sanitation Division, the Ministry of Health and Welfare, using antibiotic-sensitive strains of bacteria as test organisms (see Table 5). The microbial inhibition tests use a disk placed on an agar plate that contains an antibiotic-sensitive strain of bacteria that is incubated for a specified period of time (e.g., 17 hrs), depending on the specific assay. Although these bioassays are effective in detecting antibiotic residues, they suffer certain disadvantages including a requirement of having some technical skills and equipment, delayed results, and susceptibility to interfering substances which may yield false positives.

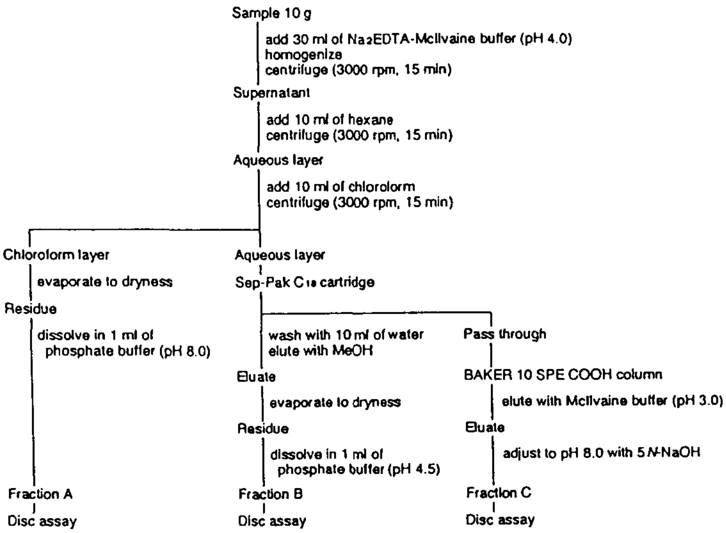
The simplified classification method for antibacterial residues, based on microbiological

Table 5. Individual Methods by Microbiological Assay for Residual Antibiotics in Livestock Products in Japan[a]

Test organisms	Antibiotics
Bacillus stearothermophilus var. *calidolactis* C-953	Ampicillin, cloxacillin, lasalosid, dicloxacillin, salinomycin
Bacillus cereus var. *mycoides* ATCC 11778	Oxytetracycline, chlortetracycline
Micrococcus flavus ATCC 10240	Bacitracin
Micrococcus luteus ATCC 9341	Tylosin, spiramycin, kitasamycin, oleandomycin, erythromycin, penicillin G
Bacillus subtilis ATCC 6633	Kanamycin, Streptomycin, enramycin, monensin
Bacillus cereus ATCC 19637	Flavophospholipol
Pseudomonas syringae X 205	Hygromycin B
Corynebacterium xerosis NCTC 9755	Thiopeptin, virginiamycin
Bordetella bronchiseptica ATCC 4617	Colistin
Bacillus brevis ATCC 8185	Destomycin A
Staphylococcus epidermidis ATCC 12228	Fradiomycin, novobiocin
Escherichia coli NIHG	Chloramphenicol

[a] Announced Analytical Methods for Residual Substances in Livestock Products, Vol. 1, Veterinary Sanitation Division, Environmental Health Bureau, Ministry of Health and Welfare, Japan.

assay, was published in 1991[6]. Scheme I shows that this method applies a pulp disc technique using *Bacillus subtilis* (ATCC 6633), *Micrococcus luteus* (ATCC 9341), and *Bacillus cereus* var. *mycoides* (ATCC 11778), in identifying antibacterial residues in fractions obtained by chemical separation. Fraction A contained macrolide antibiotics (MLs) and sulfa drugs (SAs), fraction B contained tetracyclines (TCs) and penicillins (PCs), and fraction C contained aminoglycoside antibiotics (AGs). Antibacterial agents detected in each fraction were confirmed by the different patterns of growth inhibition of *B. subtilis*, *M. luteus*, and *B. cereus* (see Table 6). The purpose of a screening test is to quickly determine whether or not an analyte is present at or near the level



Scheme 1. Microbiological assay for antibiotics in livestock products.

Table 6. Classification of Antibacterial Agents in Fractions by Inhibition Patterns of Test Organism

Fraction	Test organism			Determination of antibacterial agent
	B. subtilis	*M. luteus*	*B. cereus*	
A	+	+++	−	MLs
A	+++	−	−	SAs
B	+	−	+++	TCs
B	+	+++	−	PCs
C	+++	−	+	AGs

Source: See reference[7].

of concern in the target sample. While the development of analytical methods has made it possible to detect residual drugs at trace levels, the detection limit required for each drug in livestock products should be defined by a safety evaluation based on the toxicological aspects. So far, a level of 50 parts per billion (ppb) has been considered suitable for a provisional detection limit for the analysis of most drugs used in Japan. Chromatographic methods of analysis for antibiotics in animal tissues and foods have been the subject of several recent reviews[7-9] and are dealt with in detail in chapters 5–11.

Various chemical techniques have been developed for the analysis of residual antibacterials using spectrophotometric procedures, thin-layer chromatography (TLC), enzyme immunoassay (EIA), gas chromatography (GC), high-performance liquid chromatography (LC), and mass spectrometry (MS)[10,11]. Volume II contains approved chemical methods based on TLC, GC and spectrophotometry for synthetic antibacterials[12]. When using the methods in Volume II, some drugs cannot be detected with sufficient sensitivity to measure trace amounts and to simultaneously determine each individual drug, so the government has proposed a multiresidue analytical method using LC[13].

After reading recent reviews[5,10-12], it is clear that using LC with a UV detector has been widely applied for the routine analysis of residuals of veterinary drugs, and that it is more accepted than GC techniques for these analytes. However, when the peak of a target drug has appeared on the LC chromatogram, conventional LC methods lack qualitative information. It is necessary to ensure the identification of the observed peak before taking regulatory action for food hygiene. Although a few GC-MS methods have been developed to confirm target drugs, these methods are complicated and time consuming, requiring suitable volatile derivatives to be prepared for the large-scale screening tests. For this purpose, alternative instrumental analyses using a photodiode-array detector[14,15] or LC-MS[16-19] provide several powerful approaches in identifying the drugs detected in regulatory samples. The combination of LC with photodiode-array detection and with MS holds tremendous potential for the analysis of residual synthetic antibacterials, and there is considerable urgency for developing reliable instrumental methods of analysis for antibiotics in the edible tissues of farmed livestock and fish.

Surveillance for Residues in Livestock Products

Surveillance of food for chemical contaminants is carried out in many countries in order to ensure that the human diet is safe[20,21]. Table 7 shows the results of a national survey conducted by the Veterinary Sanitation Division, Environmental Health Bureau, Ministry of Health and Welfare, on residual antibiotics in domestic meat and fish in Japan, for the fiscal years 1990–1992. The monitoring samples collected at the urban and rural prefectures are analyzed by the laboratories of government authorities such as Meat Inspection Offices, Market Food Inspection

Table 7. Antibiotics Residues in Domestic Meat and Fish in Japan

Fiscal year	Sample number test	Meat			Fish										Egg	Honey	Tot.
		Beef	Pork	Ch.	Y	Bream	Salmon	Carp	Trout	Ayu[a]	Eel	Mack-erel	Tilapia	Flat-fish			
1990	Total	594	910	301	40	72	1	53	38	12	67	—	—	—	—	137	2,224
	Penicillins	(1)	(3)	—	—	—	—	—	—	—	—	—	—	—	—	(2)	(6)
	Tetracyclines	—	(13)	—	—	—	—	—	—	—	—	—	—	—	—	(13)	(26)
1991	Total	1,866	4,974	612	88	83	10	61	73	51	93	1	13	10	341	210	8,486
	Penicillins	(5)	(7)	—	—	—	—	—	—	—	—	—	—	—	—	—	(12)
	Tetracyclines	—	(5)	(1)	—	—	—	—	—	—	—	—	—	—	(1)	(8)	(15)
	Aminoglycosides	—	(1)	—	—	—	—	—	—	—	—	—	—	—	—	—	(1)
1992	Total	1,939	3,063	3,116	103	134	20	57	86	118	128	4	14	20	608	354	9,764
	Penicillins	(1)	(1)	—	—	—	—	—	—	—	—	—	—	—	—	—	(2)
	Tetracyclines	—	(9)	(1)	—	—	—	—	—	—	—	—	—	—	—	(7)	(17)

Ch.: Chicken; Y: Yellowtail, Tot.: Total. The numbers in parenthesis indicate the detected number.
[a] Ayu means sweetfish in Japanese.

Offices, and Institutes of Public Health. As was seen for the three year surveillance program using the approved biological assay, the livestock products available to the public contain a relatively low incidence of antibiotic residues.

Recent Developments

According to the Japanese Food Sanitation Law, food should not contain antibiotics and synthetic antibacterial substances. In order to monitor the drug residue levels in livestock products, simple and reliable methods are required. In addition to developing improved methods for residual analysis, evaluating veterinary drug residues should include the parent compounds and their metabolites in any edible portion of the animal product. The development of new veterinary drugs must also be evaluated based on the drugs' efficacy, their safety to the intended animal species, and their safety to the humans consuming animal products. Moreover, although animal drug residues in livestock do not appear to be a problem, it is necessary to monitor the various products by appropriate methods to ensure continued adherence to food hygiene standards.

In January 1994, the Japanese Health and Welfare Ministry requested the Food Hygiene Investigative Committee to determine maximum residue limits for those antibiotics, antibacterial agents, and hormones that have not been banned. According to the Japanese Food Sanitation Law, there is no legal regulation on anthelmintic residues in various livestock. However, in addition to antibacterial agents and hormones, tolerances are to be set for residual anthelmintics.

In Japan, approximately 30 anthelmintics are used to prevent and cure parasitosis from trematodes, nematodes, cestodes, and mites in fowl, swine, beef and dairy cattle, sheep, goats, horses, and honeybees. The anthelmintics used are synthetic antibacterials, benzimidazoles, organophosphorates, phenols, salicylanilides, and three antibiotics including destomycin A, hygromycin B, and ivermectin. Tolerances established for these compounds must be based on a consideration of the toxicological data and the establishment of a no-effect level (NOEL) for that substance in an experimental animal. As lower acceptable residue concentrations are recommended, it will be necessary to ensure the availability of a practical analytical method suitable for routine residue analysis at the limit established for the target drug in edible tissues.

The diffeent methodological approaches, which include both microbiological assays and the current range of chemical analysis techniques such as LC with photodiode-array detection, GC-MS, and LC-MS, are powerful and useful for residual analysis. However, complete facilities for residue analysis using all such methods are not available in all regulatory laboratories. Although LC analyses are currently only used for official residual analysis of synthetic antibacterials, the development of reliable LC methods for antibiotics will help overcome these problems. The recruitment and promotion of talented analysts is an essential element in developing a successful program.

Conclusion

As the scale of production expands, the production cost declines and the time required for upkeep diminishes. There is a movement away from family-run farms to more efficient, large-scale operations managed as a company. Although the livestock industry has grown to meet the increased demand, the moderation of consumer demand and deregulation of beef imports have created a need for livestock producers to improve their business foundation.

Even though the incidence of antibiotic residues in foods of animal origin remains low and the human health risks associated with these residues are small compared with other food safety problems, the public's attention has focused on the residue issue. The establishment of international standards that ensure human food safety and the harmonization of international trade in animal products is necessary. Studies for the safety evaluation of veterinary drugs in food must be continued with respect to pharmacological, toxicological, and chemical aspects. In particular, the effects of residual antibiotics in foods on consumers, e.g. allergic reactions, or the induction of resistant bacteria must be investigated.

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